1968

The Effect of Phytohemagglutinin on Skin Allograft Survival in Mice

Clark D. Moore
Loyola University Chicago

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Master's Thesis

May 23, 1968

by

Clark D. Moore

Department of Microbiology

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Abstract

Single or repetitive i.p. injections of Phytohemagglutinin (PHA) induced a selective marked decrease in blood and bone marrow lymphocytes in mice, suggesting that PHA might have a specific immunosuppressive effect. To test this hypothesis, parallel experiments were conducted using female C57Bl/6J (H-2b) mice as recipients and either female C3H/HeJ (H-2k) or female (C57Blx C3He) F_1 (H-2b,k) hybrid mice as donors of full-thickness skin grafts. Immunosuppression, as indicated by prolonged graft life, was induced by as little as a single injection of 0.1 mg and increased with the dose to the maximum tolerated dose of 3.0 mg PHA. Several time and dose responses were tested. Maximal immunosuppression was induced using a regimen of 3.0 mg i.p. 24 hrs. before grafting, followed by daily injection of 0.5 mg PHA i.p. These results lend support to the hypothesis that PHA, like anti-lymphocyte serum, has a specific suppressive effect on cell-mediated immunity.

Introduction

Renaissance painters have depicted attempts to allograft human tissues which occurred as early as the fourth century A.D. Through the years, many individuals have attempted grafts of heterogeneic (interspecies), allogeneic intraspecies), syngeneic (among identical twins or members of inbred strains) and autogeneic (within the same individual) materials with variable success. Advances in the fields of immunology and surgery within the last 25 years, however, have made it possible to transplant a full spectrum of organs and tissues. Allografts of bone, cartilage and blood vessels are now routinely performed. Grafts of this nature, however, are unique in that none of these three types of tissue is substantially antigenic (10,19). Sophisticated surgical techniques allow even the replantation of severed limbs and digits now with retention of at least partial function of the appendage (54). Further, it is technically possible to experimentally transplant a diverse spectrum of organs such as kidney, lung, liver, heart, pancreas, stomach and various endocrine glands (5,17,20,49,55,59). Clinically, though, only kidney allografts are routinely performed.

Extensive work on the genetics of transplantation has shown that the success of transplants is determined by multiple histocomatibility genes designated by the letter H. These H genes express themselves as alloantigens, located primarily on cell surfaces, which incite an immune response when transferred into a host with antigenic specificities differing from those of the donor animal. In mice, analysis by linkage tests employing marker genes, and analysis by transplants from parental strains to their F_1-hybrid offspring have shown that there are at least 15 H loci. Further, these have

been shown to be codominant, i.e., individually expressed in the heterozygote. The loci are widely distributed throughout the chromosomes and the majority of available evidence indicates that they are multiple allelic systems with many possible antigenic specificities for each locus. Histocompatibility loci also exhibit varying immunogenicity or strength; most impose only weak barriers to transplantation and are easily overcome or are not expressed on some genetic backgrounds. The H-2 locus, an extremely complex system of multiple alleles, presents the strongest transplantation barrier known in mice, and is seldom overcome even by tumor transplants (23). Familial studies of antigen distribution indicate that the situation in humans is analogous to that in mice (3,11), and definitive tests of histocompatibility have been developed which allow close matching of donors to recipients.

Two factors remain the major obstacles preventing successful allografting of tissues and organs. Procedures for obtaining and preserving donor tissues for subsequent transplantation must first be improved. To this end, the use of cadaver donors seems to be the most promising; through the use of organ perfusion with chilled Ringer's solutions and storage at low temperatures, the period of viability of many organs has been greatly extended and the destructive effects of post-mortem anoxia and autolysis minimized. These techniques now allow the use of cadaver kidney for transplantation and are being established for use with other organs (27). The second factor is the problem of inducing specific immune suppression of the response to allogeneic materials without producing undesirable side effects. This difficulty in inducing specific immunosuppression is a consequence of the complex mechanism

of graft rejection and of the diffuse nature of the lymphoid and reticulo-endothelial systems. According to Elves (12), the development of blood vessels between the graft and host during rejection of allografts is followed by migration of host mononuclear and neutrophil leucocytes into the graft. These cells include a high proportion of small lymphocytes which become sensitized to the graft antigens probably by either of two means: (a) direct interaction with soluble antigens that the graft has shed, or (b) indirectly by means of an RNA-like transfer factor liberated by macrophages which have ingested and processed antigenic graft materials. These sensitized lymphocytes then either leave the graft via the afferent lymphatics to the regional lymph nodes where they instigate a proliferative reaction resulting in the production of first more sensitized lymphocytes and finally antibody-containing plasma cells, or they remain in the graft where at some stage in rejection they probably transform into large pyrononophilic cells which have the ability to divide. It is likely that following division, these cells become mature plasma cells--this latter cell type appearing in large amounts when the graft is already necrotic. The small lymphocytes formed in the lymph nodes are released into the lymphatics and circulation from which some return to the graft and participate in the rejection reaction. From <u>in vitro</u> experiments, it appears that rejection is the result of direct cytocidal action on the part of the small lymphocytes, which may also contribute to the rejection by occluding the afferent blood supply to the graft. The plasma cells which appear in the lymph nodes and graft not only contain antibody to the graft donor's antigens, but are probably synthesizing it. This antibody seems, however, to play only a minor role in the allograft response; titers

very often rise only upon rejection of the graft, and such rises are unpredictable. It is most likely that this antibody plays only an opsonic role and in itself is not a sufficient cause for graft rejection. Recently, Rapaport et al. (43,44) have reported that allografting is associated with a marked rise in the level of circulating heterophile antibodies. They suggested on this basis that allograft rejection may be partly the result of an immune response to heterophile antigens in the graft tissue. This finding has also opened new alleys of investigation on the problem of autoimmunity. Further, lymphocytes and macrophages are widely distributed throughout the tissues of the body, necessitating that a systemic rather than localized treatment be used to achieve immunosuppression.

The agents currently in use for inducing immunosuppression leave much to be desired because of their lack of specificity of suppression and the extent of their side effects. There are, according to Berenbaum (4) and Schwartz (47), four means by which immunosuppressive agents can act: enzyme inhibition, template damage, structural damage, and mitotic inhibition. Accomplishment of any one or a combination of these effects leads to reduced, unbalanced, or misdirected synthesis of cellular components and ultimately to cell death. The agents currently preferred, such as azathioprine, prednisone, actinomycin-C, and X-irradiation, accomplish their purpose primarily as a result of the selectively greater sensitivity of the lymphoid system compared to other tissues rather than as a result of any specifically unique sensitivity of the lymphoid system to these agents. In addition, Starzyl (51) has shown that, in kidney transplant patients, the patient with a rejection crisis and thus in most need of effective immunosuppressive therapy is also the most suscept-

ible to the induction of irreversible bone marrow failure leading to infection and death.

Another possible approach to immunosuppression is chronic thoracic duct drainage, a means of specifically eliminating or depleting the immunologically competent cells (i.e., small lymphocytes) and thereby rendering the host unreactive to allografts. Among others, Woodruff (64) through animal experiments and Tilner et al. (56) through human therapy have shown this approach to be effective in delaying the onset of the allograft response. A disturbing note, however, is the observation that once rejection begins, it proceeds with the same rapidity and severity as an unimpaired response. This phenomenon and the limited extension of graft life obtained has limited the value of lymphocyte depletion as a primary means of specific immune suppression, but its value as an adjunct to other means is indicated by its additive effect on azathioprine-prednisone therapy in human renal allografting.

To date, the only promising and apparently specific agent is anti-lymphocyte serum (ALS). Many investigators have experimentally demonstrated the substantial suppressive effect of this agent on allograft immunity (22, 29). On the other hand, considerable problems have been encountered in the use of ALS. Most conspicuous of these are the lack of a reliable test *in vitro* which correlates with *in vivo* effect and the problem of specificity of the ALS. A third problem is the side effects associated with protracted administration of the antiserum. There are four *in vitro* tests of ALS which have been extensively studied: (a) RBC agglutination, (b) lymphocyte agglutination, (c) lymphocyte cytotoxicity (in presence of complement), and (d) lymphocyte transformation (RNA and DNA synthesis). As yet, none of these

tests has been shown to reliably predict the in vivo immunosuppressive effect of ALS (63). Antigens with which ALS reacts are not only found in lymphocytes but are present in cells widely distributed throughout the body. It has been shown that even mouse tail skin epidermal cells can be used to elicit an active ALS. Seegal et al. (48) have observed a Masugi-type nephritis presumably due to rapid binding of antibody to the glomeruli of the transplanted kidneys. Thus, the administration of ALS to prolong the life of allografts can lead to their eventual destruction as a result of antigens common to both graft tissue and lymphocytes. Serum sickness and anaphylactic reactions have complicated protracted administration of ALS and the eventual immune response to the foreign proteins of heterologous antisera limit the length of time for which they can be administered (30). The purification and use of anti-lymphocyte globulin (ALG) which is much less immunogenic than whole serum, however, has to an extent reduced these problems (63). Levey and Medawar have summarized in 12 points our present state of knowledge concerning anti-lymphocyte serum:

1) ALS has the character of an antibody
2) ALS does not act by enhancement
3) The activity of ALS resides predominantly in the 7S fraction
4) ALS is not directed against antigens peculiar to lymphoid cells
5) Lymphoid cells are the effective targets of ALS
6) ALS does not act through lymphocytic depletion
7) The state of unresponsiveness induced by ALS is not immunological tolerance

8) The immunosuppressive action of ALS is not a by-product of some more generalized organic damage

9) The immunosuppressive effect of ALS outlives its own metabolic lifetime

10) The effect of ALS on lymphoid cells persists through at least one cell division

11) ALS is particularly effective in the homograft reaction

12) ALS may act in the first instance on peripheral lymphocytes

The attention of many investigators, particularly in the past few years, has been drawn to phytohemagglutinin (PHA) in the expectation that this compound may have a specific suppressive effect on the allograft response in a manner similar to that of anti-lymphocyte serum. An aqueous extract of the leguminous plant Phaseolus vulgaris, PHA and similarly obtained extracts from other plants of this genus have been known to researchers since Ehrlich used them in his studies of antigen-antibody interactions.

There are two forms of Difco PHA (PHA-P and PHA-M) and a Burroughs-Wellcome PHA which are available commercially. Rigas and Osgood (45) first described the extraction of PHA-M from the red kidney bean in 1955 and reported it to be a mucoprotein containing approximately 50% carbohydrate. Pharmacologic activity was associated with the protein and in 1964, Rigas and Johnson (46) outlined the procedure for dissociating the protein moiety from the bulk of the polysaccharide under acid conditions. This preparation, which they labelled PHA-P, has approximately 50 times the erythro-agglutinating activity of PHA-M. It has been determined experimentally by this investigator that this proportionality is also true with respect to the

induction of transformation of human lymphocytes in vitro and either the i.v. or i.p. LD50/48 hrs. PHA-P has been further shown to have a sedimentation coefficient of 6.5S, a diffusion coefficient of 4.8 cm^2 $sec^{-1}x10^{-7}$, a molecular weight of 128,000 and an isoelectric point of pH 6.5. It is poor in sulfur-containing amino acids and rich in aspartic acid plus asparagine, serine, threonine and leucine; it has alanine as the only N-terminal amino acid; it has a carbohydrate content of 3.4%. It is suspected but has not been definitely shown that this carbohydrate is covalently linked to the protein moiety. There are at least 23 varieties of Phaseolus vulgaris, all of which yield hemagglutinins of somewhat similar activity upon aqueous extraction. Although the very great majority of work in vitro and in vivo with both humans and animals has been carried out with the extract of the red kidney bean, occasional reports have been published using the phytohemagglutinins from other varieties of Phaseolus vulgaris. These preparations all exert somewhat similar agglutinating activities, but they differ considerably in their toxicities and physical properties and may also differ in their mitogenic activities (53). Previous work from this laboratory has further shown that PHA-P varies considerably in activity from lot to lot as measured by the in vitro transformation of lymphocytes.

In 1929 Goddard and Mandel published work which involved possible in vivo toxic effects of PHA in rabbits (21). Interest in PHA, however, remained only in its agglutinating properties as a tool in preparing cell-free sera until 1960 when Nowell reported the mitogenic effect of PHA on peripheral blood leucocytes in vitro (40). Since then, several investigators have described the blastoid transformation of lymphocytes induced in vitro

by this substance (8,34). In addition, this blastoid transformation has been shown to be preceeded by an exponential acceleration of RNA, protein, and DNA synthesis. Bach and Hirschhorn (1) have reported a rise in the gamma globulin content of lymphocytic cells resulting from treatment in vitro of human lymphocytes with phytohemagglutinin. They suggested from this that this morphologic transformation of lymphocytes arises as an immunologic reaction to PHA acting as a non-specific antigen and stimulating antibody production. Similarly, Elves et al. (14) reported detectable amounts of gamma globulin arising in transforming human small lymphocytes subsequent to treatment in vitro with PHA or antigens. Since then, however, other authors have demonstrated that PHA stimulates all classes of RNA and protein and DNA (32,36) suggesting that the lymphocytes' response to PHA differs biochemically from their response to antigens. It is interesting that, as mentioned above, anti-lymphocyte serum has also been shown to induce blastoid transformation in peripheral blood lymphocytes in vitro even though this property of ALS is not a requisite for immunosuppression in vivo (63).

Phytohemagglutinin has been used in patients with aplastic anemia in the attempt to restore their bone marrow cellularity (16,26,60). PHA has also been used in the Veterans Administration Hospital, Hines, Illinois on two patients with bronchogenic carcinoma treated by external irradiation (52). Few experiments have been conducted on laboratory animals in the attempt of proving the validity of the clinical treatment. Elves et al. (15) failed to demonstrate any consistent effects in rats after one or repetitive i.v. or i.p. injections of PHA. Papac (41) reported that mice and rats exposed to varying doses of irradiation failed to show stimulation of marrow regener-

ation after treatment with the highest tolerated doses of PHA administered i.v.

The possibility of an immunosuppressive effect of phytohemagglutinin has been previously suggested by the work of Calne and Wheeler (9). In a preliminary report, they observed following combined treatment with PHA and azathioprine a moderate prolongation of the life of renal allografts. These authors noted a slight immunosuppressive activity of PHA alone and a potentiation of the effect of the Imuran. In addition, two recent papers, one by Spreafico et al. (50) and the second by Elves (13), have shown that phytohemagglutinin can suppress antibody synthesis in vivo. Following the i.v. administration of this compound to rats, these authors noted a decrease in the amount of hemolytic antibody formed during the primary and secondary immune response to chicken or sheep red blood cells.

Using skin allograft rejection in mice as a marker of immune competence, the present work was undertaken to answer the following three specific questions:

1) What is the effect of phytohemagglutinin on cell-mediated immunity as measured by prolongation of the life of mouse skin allografts following i.p. administration of PHA?
2) What is the optimal dose and most effective schedule of administration of this preparation in prolonging mouse skin allograft life?
3) What is the relationship of the in vivo effects of PHA to the in vitro action of this compound?

Material and Methods

Animals. Adult female CFI mice eight to ten weeks of age (Carworth Farms Inc.) were used for the blood and bone marrow studies. It was felt that a disparity across the H-2 locus would provide the most definitive test of immunosuppression. Accordingly, adult female C3H/HeJ (H-2b) mice eight to ten weeks of age and adult female C57BL/6J (H-2k) mice of the same age (Roscoe B. Jackson Memorial Laboratories) were used for these experiments. Also used in this study were eight to ten weeks old adult female (C3H x C57) F_1 (H-2b,k) hybrid mice bred from the above stocks.

Phytohemagglutinin. Each vial containing 100 mg phytohemagglutinin-P (PHA-P, Difco lot nos. 497992 and 496843) was diluted with 5.0 ml sterile isotonic saline. For the several doses of PHA used in this study, dilutions were made with sterile isotonic saline to the desired amounts of PHA per 0.5 cc, the amount of fluid injected i.p.

Absolute and differential counts. Blood samples were taken from the tail veins of mice. Smears were made, stained with Wright's stain, and 100 cells counted to establish a differential count. According to standard hematologic procedure (61), blood samples were diluted in a WBC serologic pipette with a 3% solution of HCl and New Methylene Blue to hemolyze the RBC and counted in a hemacytometer to determine the absolute WBC. From these two counts, the absolute number of lymphocytes was calculated. Blasts and mitoses in the bone marrow were counted as part of the differential.

Skin grafting. Adopted for this tudy was a modification of the method described by Medawar using full-thickness skin grafts (33). Twenty-four hours before grafting, the donor animal and prospective recipients were

closely shaved with an electric clippers, number 40 head. Immediately prior to grafting, the donor animal was sacrificed by cervical dislocation and skinned using aseptic technique. The skin was then stretched out inner side up at even tension, flushed with sterile saline, scraped free of fat and fascia with a sterile number 11 scalpel, and from 20 to 30 circular grafts 1.0 cm in diameter were cut using a sterile cork borer. These grafts were then placed between filter paper wet with sterile saline at 4^{o} C until use. While being held in the investigator's left hand, each recipient mouse was lightly anesthetized using an ether nose cone, and a bloodless circular graft bed to the level of the panniculus carnosus was prepared on the ventral chest wall using a small, sharply pointed, curved scissors. First, make a nick at the posterior end of the bed site with the point of the scissors. Next, reverse the scissors and free the skin from the underlying tissue by sliding them through the nick and under the skin then spreading the points slightly. Lastly, cut a circular piece of skin 1 cm in diameter to expose the panniculus carnosus. The graft was placed in position, rotated 180^{o} from normal hair direction, and the edges of the graft apposed evenly and symmetrically to the edges of the bed. No sutures were used; the graft was held in place by means of an overlay of vaseline impregnated gauze and a dressing of adhesive tape. The animals were housed two per cage for seven days postoperatively, at which time the dressings were removed and a loose dressing of gauze and scotch tape placed on the animal for 24 hours. When this final dressing was removed, the animals were housed five per cage and examined daily for evidence of graft rejection.

Graft rejection. Complete rejection of the graft was taken as the end

point of each experiment. This was determined by visual inspection of the grafts in situ according to criteria established by Billingham et al. (6).

Experimental procedure. Each experiment was designed and carried out as discussed in the results. Each point on the charts of the experimental graft groups represents 15 to 20 mice (average graft survival ± one S.D.) and there are 30 to 40 mice represented by the plots of control graft survival ± one S.D. All blood and bone marrow studies are shown with 95% confidence limits. Each experimental group contains at least 5 animals and the control groups contain 10 to 15 animals. No animals were bled more than one time.

Results

The first experiment was designed to test the effect of a single 3.0 mg dose of PHA as a function of time between administration of the PHA and grafting. As can be seen in Figure 1, PHA given from 30 minutes after to three days before grafting induces in the C3H to C57 system a significantly longer graft survival (10.4 days S.D. $\pm$ 1.0) compared to the control graft survival (8.3 days S.D. $\pm$ 0.4). PHA had only a small effect on graft survival if given four days before grafting--the period of time shown in Figure 2 to correspond with the time of lymphopenia induced in CF1 mice by the same dose of PHA.

Next, the effect of daily repetitive injections of 1.5 mg PHA i.p. was tested. Again, a better graft survival was noted in the PHA treated group than in the control group, seen in Figure 3. However, a slightly better graft survival followed the single injection of PHA (10.5 days S.D. $\pm$ 0.7) rather than the repetitive injections, decreasing to a minimum value in the four injection group (9.5 days S.D. $\pm$ 0.8).

Several experiments were then performed to investigate the dose-dependence of skin graft life following a single injection of PHA i.p. In these experiments, both the C3H to C57 and the F_1-hybrid to C57systems were used. Figure 4 shows that the average graft life was significantly prolonged in the C3H system by treatment with PHA, but did not vary significantly over the dose range tested (10.3 days S.D. $\pm$ 0.5). Even the small dose of 0.1 mg significantly increased the graft survival (10.2 days S.D. $\pm$ 0.4) as compared to the survival of control grafts (8.3 days S.D. $\pm$ 0.4). A different response was observed in the F_1-hybrid to C57 system. Here there was a direct correl-

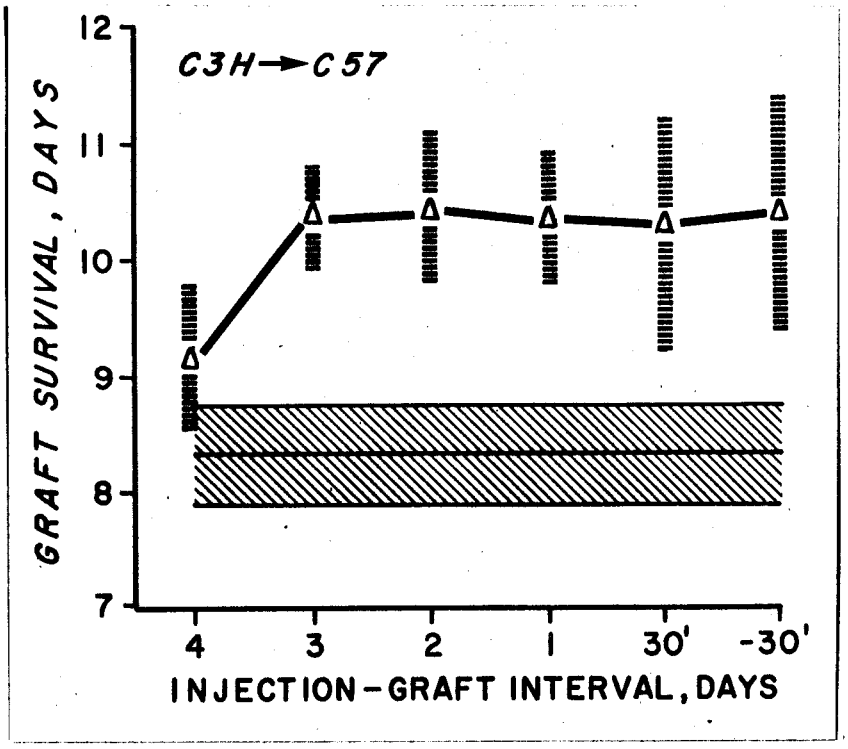

Figure 1. Skin graft survival as a function of time after one i.p. injection of 3 mg PHA-P. All values ± one S.D. Control survival shown in shaded area.

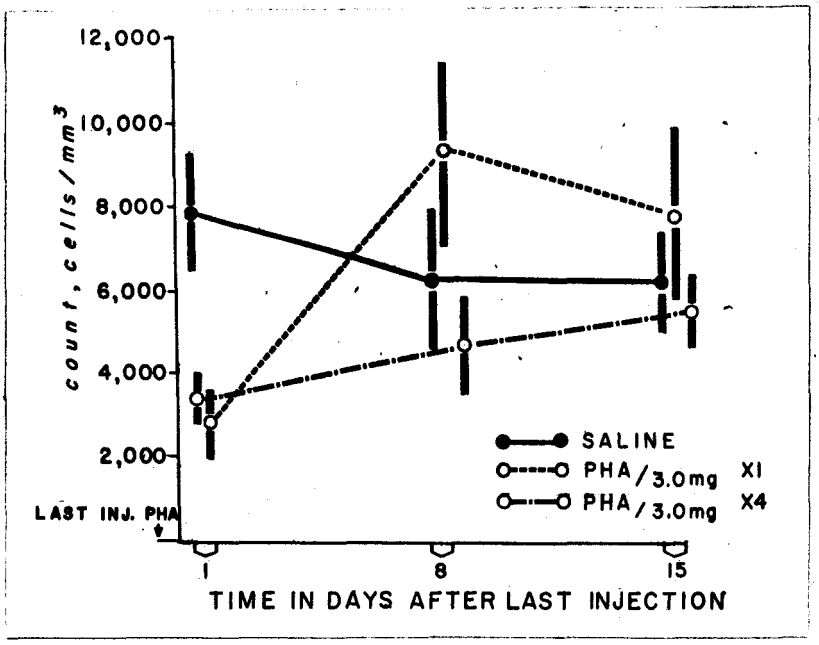

Figure 2. Absolute blood lymphocyte count in CF1 mice after i.p. injection of PHA-P. 95% confidence limits shown.

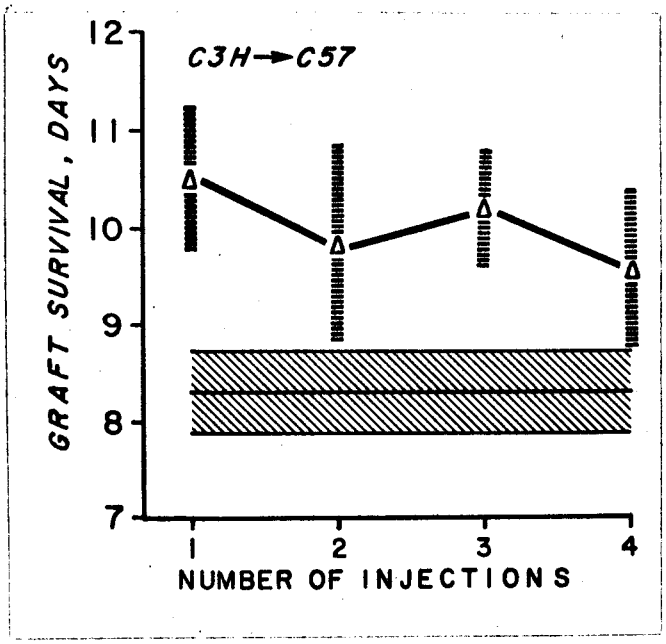

Figure 3. Survival of skin grafts performed 24 hours after the last daily injection of PHA-P 1.5 mg. All values ± one S.D.

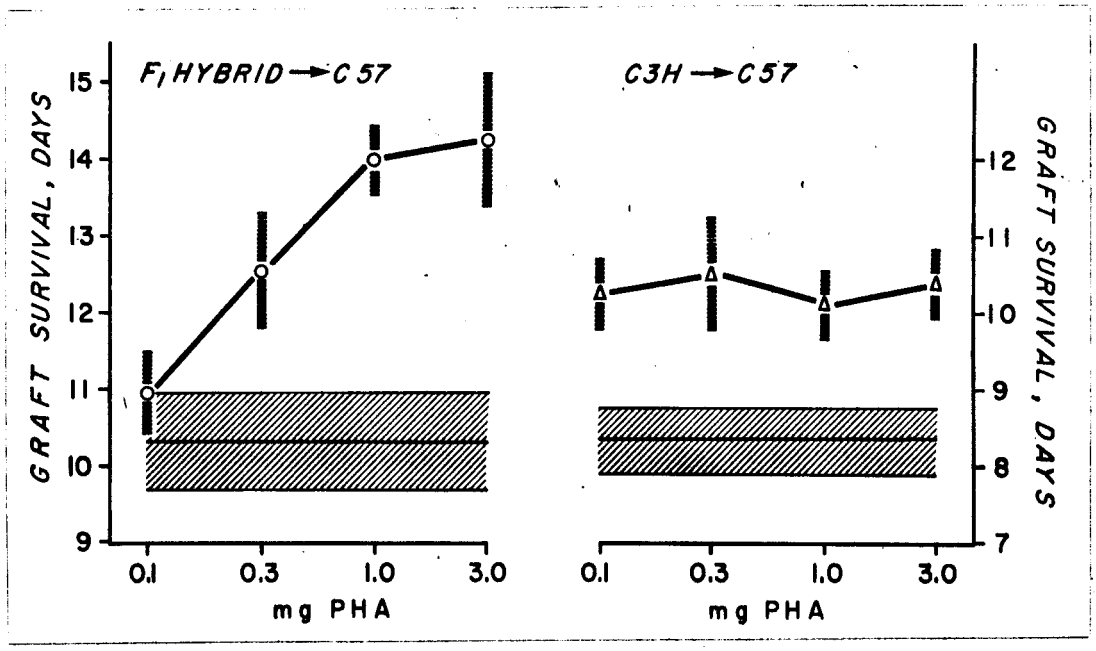

Figure 4. Survival of skin grafts performed 24 hours after a single injection of PHA-P as a function of dose. All values ± one S.D.

ation between dose and survival, higher doses affording longer survival. No correlation between graft survival time and peripheral blood lymphopenia was observed in this experiment. Table 1 relates the changes in absolute blood lymphocyte levels to graft survival and dose of PHA used. It is apparent from this table that the immunosuppressive effect of PHA is not dependent upon the production of peripheral blood lymphopenia. A further observation is that the relative percentage of blasts and mitoses in the bone marrow of CF1 mice following a single injection of 3.0 mg PHA i.p. (Fig. 5) rise concommitantly with the changes in the peripheral blood lymphocyte level.

Maximal prolongation of allograft survival has been obtained with the following treatment. First, one injection of 3.0 mg PHA was given intraperitoneally 24 hrs. before grafting. The graft was followed by daily injections of 0.5 mg PHA starting one day postoperatively and extending from two injections up to 14 injections in the C3H system or 24 injections in the F_1-hybrid system, i.e., the time of complete rejection in the two systems. Plotting graft survival against the number of daily injections, Figure 6 shows that there is again a disproportionate response in the C3H system with respect to small doses of PHA while there is an approximately proportionate response in the F_1-hybrid system. In both systems, however, the maximum graft life obtained is approximately 100% better than the control (14.0 days S.D. $\pm$ 0.9 versus 8.3 days S.D. $\pm$ 0.4 with C3H donors, and 23.9 days S.D. $\pm$ 1.2 versus 10.3 days S.D. $\pm$ 0.6 with F_1-hybrid donors). In all instances the survival of grafts in the control group were significantly less than those in the treated groups.

It is interesting that the degree and rapidity of rejection, once begun,

Table 1.

COMPARISON OF GRAFT SURVIVAL AND ABSOLUTE LYMPHOCYTE LEVELS AS A FUNCTION OF SINGLE DOSE OF PHA-P I.P.

Dose PHA mg. I.P.	Prolongation Graft Life %		Absolute Blood Lymphocyte Count cells/mm^3		
	C3H	F_1	2 days	4 days	7 days
0.1	22.8	5.6	9,500	14,500	4,500
0.3	26.5	22.3	12,200	15,850	10,900
1.0	21.7	35.9	8,550	9,750	9,350
3.0	25.3	38.8	2,800	3,950	9,200
N.T.	-	-		6,600 ± 2,500	

C57BL/6 female mice were used for this blood study. No animal was used more than once; there are five mice represented by each average value. The control value is an average of 15 mice, 5 bled on each of the three days shown, and the 95% confidence limit is given.

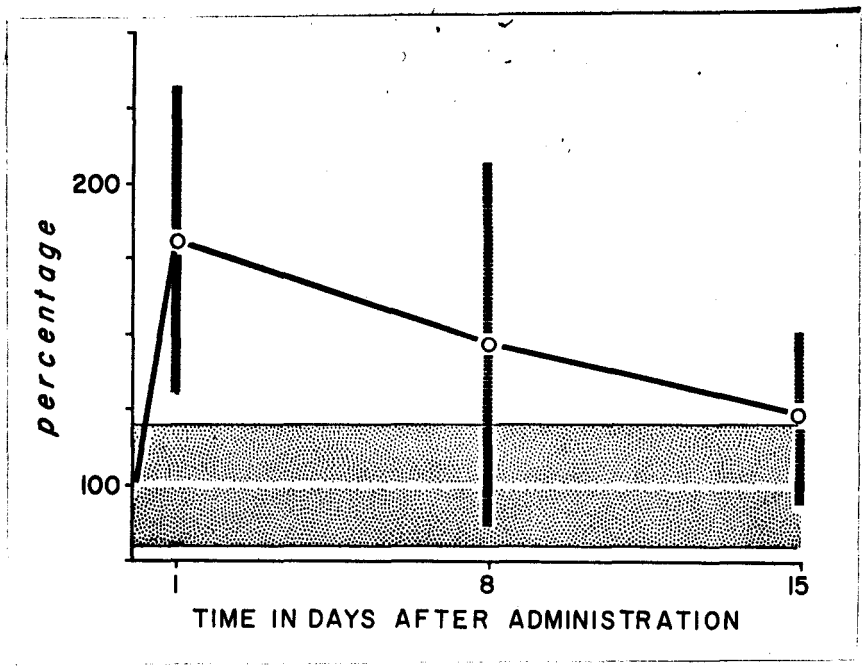

Figure 5. Percentage change in the relative number of bone marrow blasts after i.p. injection of 3.0 mg PHA-P in female CF1 mice. 95% confidence limits are shown.

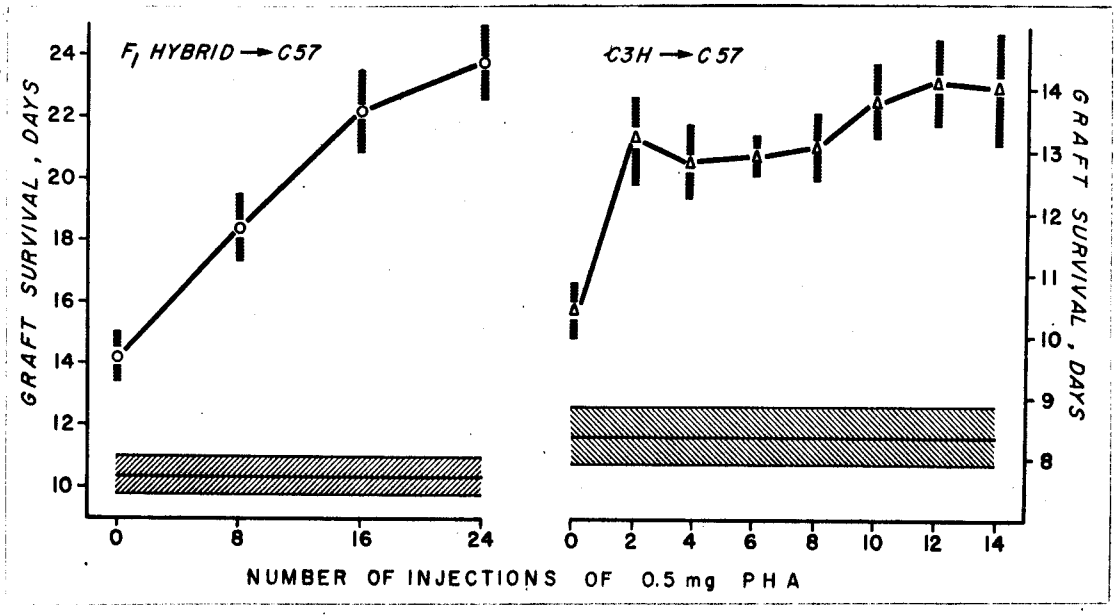

Figure 6. Survival of skin grafts performed 24 hours after 3 mg PHA-P and 24 hours before 0.5 mg daily. All values ± one S.D.

was unaffected by the PHA treatment, and resembled the rejection process in the control animals. Further, in all experiments, no deaths could be attributed to the toxicity of PHA. Extensive work with 12 week old female CF1 mice has shown that the LD50 at 48 hrs. for PHA-P injected intraperitoneally is 9.5 mg. Due to the differences in weight between the CF1 and C57BL strains, this corresponds to an LD50 of 6.0 mg for the eight to ten week old C57BL mice used as graft recipients.

Discussion

Extensive investigation of alternate methods of skin grafting found them to be unsuitable for the purposes of this study before the procedure described above was adopted. This method is recommended by many considerations. The technique is quite simple and no sutures are needed to position the graft. This results in practically eliminating the problem of infection due to experimental procedures. Second, this technique is reliably and quickly performed; less than 10% of the animals have to be discarded as a result of technical error such as uncontrolled bleeding in the graft bed or movement of the graft once it is in place. Third, many grafts can be obtained from a single animal, thereby minimizing the possibility of antigenic variation being introduced by large numbers of donor animals. Fourth, there is no loss or alteration of graft antigenicity for periods of time up to 6 hrs. as a result of the methods used to prepare and store the skin grafts until use (62). Fifth, the uniform graft size insures both a more uniform antigenic challenge to the recipient animal and a consequently more uniform immune response to the graft antigens. Galton (18) has shown that with grafts from F_1-hybrids to either parent or with grafts across non-H-2 loci, larger grafts have slightly shorter survival times, indicating that in these systems, at least, the antigenic dose to which the animal is exposed can be a significant variable.

In the above experiments, PHA-P injected intraperitoneally has been shown to increase the survival of skin allografts in mice to a maximum value of 71% in the C3H to C57 system and 132% in the F_1-hybrid to C57 system. The maximum graft survivals obtained compare well with results obtained using other current immunosuppressive agents. Hoehn (27) using A to CBA (an H-2

difference) grafts as his "strong" system and C3H to CBA (a non-H-2 difference) as his "weak" system obtained a 20% and 50% respective increase in graft survival using paired Imuran and Actinomycin C therapy. These results, however, were accompanied by a 60 to 70% mortality due to drug toxicity. Treatment with bromodeoxyuridine and thioguanine in the "strong" system resulted in a 60% increase in graft survival with a mortality of 13.3%. No animals which succumbed to the effects of the therapy were included in the calculation of graft survival. Considerably better results were achieved by Levey and Medawar (30) using anti-lymphocyte serum to prolong the life of A strain skin grafts on CBA mice. They reported a 134% prolongation of graft life as compared to control graft survival with no apparent toxic effects due to the ALS. Thus, it would seem that the results obtained with PHA compare well with results achieved through other means of chemical suppression. In addition, PHA, like anti-lymphocyte serum, exerts its effects unaccompanied by any apparent toxic side effects.

Initial studies of blood and bone marrow cell levels following treatment with PHA suggested to us that the lymphopenia produced in both the peripheral blood and bone marrow could lead to a delay in the allograft response. During the course of this study, however, it became apparent that this phenomenon was inadequate to explain the longevity of allografts following treatments with PHA over the dose range and regimens tested. Although the length of time during which prolonged graft life could be induced following a single injection of PHA corresponded to the duration of peripheral blood lymphopenia, grafts performed 30 minutes before or after injection of PHA also exhibited a similarly prolonged survival. This was interesting in that these grafts

were performed before the period of lymphopenia became evident. More revealing was the observation that graft life far outlasted the periods of lymphopenia in either the peripheral blood or bone marrow. That lymphopenia production is not the mode of action of PHA in suppressing allograft rejection was conclusively established by the dose-response experiment in which it was shown that doses of PHA which induced a pronounced lymphocytosis also induced a marked prolongation of graft survival. These results are further supported by the observation that the relative number of blasts and mitoses in the bone marrow rise following treatment with PHA. What seems likely in the light of these experiments is an explanation offered by Elves (13) who suggested that "transformation of immunologically competent cells occurs in vivo with the 'siphoning off' of such cells so that they are not available to react with the immunizing antigen", in this case the allograft. The present study supported this idea and suggested further insights into the mechanism by which the immunosuppression was induced by PHA. From the studies of peripheral blood and bone marrow following the administration of PHA, it appeared that this compound acts on two distinct populations of lymphocytes in suppressing allograft rejection. The most important action of PHA is exerted primarily on the central lymphocytes through the transformation and subsequent "siphoning off" of immunocompetent cells. This was evidenced by our own observation of the rise in the numbers of bone marrow blasts and mitoses following injection of PHA and by Elves (15) who noted an increase in the mitotic activity in the spleens of mice following injection of PHA. A secondary action of PHA, apparent only after higher doses, is the induction of lymphopenia in the peripheral blood. This effect seemed to result from either a direct cytocidal

action of PHA or enhanced phagocytosis and clearance of circulating lymphocytes due to PHA-induced leukoagglutination and was observed to make only a small addition to the prolongation of graft life accomplished by non-lymphopenia doses of PHA.

Many investigators have attempted to clarify the importance of and the interrelationships between the three observed activities of phytohemagglutinin: (a) erythroagglutination, (b) leukoagglutination, and (c) stimulation of the blastoid transformation of lymphocytes. Barkhan et al. (2), using red cell absorption, and Tunis (57), employing selective heat inactivation to eliminate the red cell agglutinin, showed that this activity of PHA was not necessary to the in vitro blastoid transformation of lymphoid cells. Early attempts at separating the white cell agglutinin from the lymphocyte mitogen failed, however, and led to speculation that the blastoid transformation induced by PHA was merely the result of cell membrane changes following agglutination (24,40). As discussed in the introduction to this thesis, subsequent work indicated that PHA may act as a non-specific antigenic challenge in inducing blastoid transformation. More extensive work has shown this theory to be an inadequate explanation. Recently, Weber et al. (58) using ion exchange chromatography to separate the leukoagglutinating and lymphocyte-stimulating activities of PHA, and Borjeson et al. (7) using Vi polysaccharide to eliminate the agglutination of leukocytes, have shown the leukocyte mitogen to be physically distinct and independent of the leukocyte and erythrocyte agglutinins. A possible explanation of the basis for the mitogenicity of PHA, although unsupported by direct experimental evidence, is that PHA may act on a basic control mechanism of RNA synthesis leading to the eventual

blastoid transformation of the lymphocyte. Incidentally, this process may be similar to the mechanism of antigenic induction of antibody synthesis as suggested by a result of Spreafico and Lerner (50) who observed that PHA, while transiently suppressing the primary and secondary hemolysin response to sheep RBC, enhanced the normal background reactivity of non-immunized mice to this antigen. The idea of PHA acting through a basic inductive step in the metabolism of the cell is not new. Mueller and LeMahieu (36) have suggested that PHA may act primarily to counteract normal repressor mechanisms regulating RNA synthesis. Recently, Rabinowitz and Dietz (42) studying the induction of lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) isozymes by PHA, concluded that "PHA acts at the two genetic loci controlling synthesis of muscle and heart-type (M and H) lactate dehydrogenase polypeptide subunits". They further stated that "the synthesis of the two isozymes of malate dehydrogenase also may be under the control of separate genes which in granulocytes are influenced by conditions of cell culture, rather than by phytohemagglutinin". Suggested as a means by which PHA accomplishes these results was that it "may act at allosteric sites on protein repressors or inducers of operator genes for the M- and H-type polypeptides.....". This postulated mechanism of action of PHA is consistent with the view that this compound parallels in many respects the action of hormones which act on many sites on a variety of functionally different proteins.

In this thesis it has been shown that PHA-P given intraperitoneally is effective in suppressing the rejection of allografts across the H-2 locus in mice. A regimen of one large dose of PHA prior to grafting followed by small daily injections of this compound has been shown to yield results comparable

to those obtained with other immunosuppressive agents. Significantly, this immunosuppression was not accompanied by any mortality or apparent toxic effects of the PHA. Studies of the blood and bone marrow suggest that the suppressive activity of PHA *in vivo* is not related to a lymphopenic effect but is probably the result of the lymphocyte mitogen. It thus appears that PHA is comparable to anti-lymphocyte serum in its effectively specific suppression of allograft rejection and its lack of toxicity in the treated animal. Comparing what is known of PHA to some of the points made by Levey and Medawar (30) concerning anti-lymphocyte serum, it can be said that:

1) Lymphoid cells are the effective targets of both PHA and ALS.
2) Neither ALS nor PHA act through lymphocytic depletion.
3) The states of unresponsiveness induced by either PHA or ALS are not immunological tolerance.
4) The immunosuppressive actions of PHA and ALS are not the by-products of some more generalized organic damage.
5) PHA, like ALS, is particularly effective in the homograft reaction, inducing only a transient suppression of the humoral immune response (38,50).
6) While ALS appears to act in the first instance on peripheral lymphocytes, experimental evidence indicates that PHA may act on the central lymphoid organs. This last point raises the interesting speculation that combined PHA-ALS therapy may be particularly effective and non-toxic in preventing allograft rejection in the clinical situation.

Acknowledgement

The author wishes to thank Dr. Stefano S. Stefani, Chief, Therapeutic Radiology Service, VA Hospital, Hines, Illinois, for his continuing confidence, encouragement and guidance throughout our acquaintanceship. I want especially to acknowledge his suggesting my research problem and his assistance at the inception of this project.

Further, I would like to thank the personnel of the Radiobiology Research Laboratory and Therapeutic Radiology Service, in particular Mr. Mel Carino, Mr. Carl Kirsh and Mrs. Irene Zielonka for their invaluable technical assistance, and Mrs. Carole Weisbecker for her patience and thoroughness in typing this thesis.

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